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PRINCIPAL INVESTIGATOR: Gail E. Sonenshein, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02118-2394

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FOREWORD

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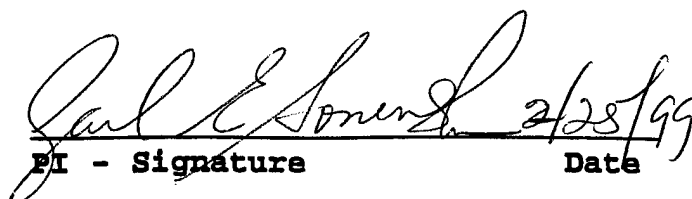
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BACKGROUND

The incidence of breast cancer has been steadily increasing over the past 50 years, and is now one of the leading causes of death among American women between the ages of 40-55 (23). In an attempt to find the reasons for this steady increase in incidence, both genetic and environmental factors are being studied. Attention has recently focused on the mechanism by which increased exposure to and bioaccumulation of these pollutants might have an etiologic role in breast cancer (13,20,38,57). The polycyclic aromatic hydrocarbons (PAHs) such as 7,12-dimethylbenz(a)-anthracene (DMBA) are specifically of interest (38). The most proximal event in PAH tumorigenesis is the binding of the chemicals to a cytosolic aromatic hydrocarbon receptor (AhR) (10,18,47). The receptor-ligand complex is translocated to the nucleus where it can bind to and alter the transcriptional level of DNA that has AhR-responsive elements. One battery of enzymes whose transcriptional induction is a hallmark of DMBA and other PAH exposure is the Phase I cytochrome P450 enzymes (9,33,38,40,59). These enzymes aid in the oxidative metabolism of both endogenous substances such as steroids, as well as in the breakdown of exogenous substances such as drugs, chemical carcinogens, and environmental pollutants. The products formed by this oxidative metabolism are often reactive oxygen intermediates (59). The potential for increased levels of oxidative stress within the cell resultant from exposure to environmental carcinogens leads us to hypothesize that this might activate expression of the NF- κ B/Rel family of transcription factors. This family of factors, which regulates transcription of multiple genes including those involved in the regulation of cell proliferation, such as the *c-myc* oncogene implicated in neoplastic transformation (17,27,31), has been found to be sensitive to the cellular redox state (rev. in 2). In agreement with this model, in preliminary experiments we have found that malignant breast cancer cell lines and primary breast cancer tissue express significant levels of constitutive nuclear NF- κ B/Rel activity. The constitutive expression of this factor suggests that NF- κ B/Rel may promote aberrant proliferation and thus play an early role in the etiology of breast cancer. A brief introduction to NF- κ B/Rel, in particular as it relates to this proposal follows.

NF- κ B/Rel Family of Transcription Factors

The transcription factor NF- κ B was first identified as a protein specific to mature B lymphocytes that interacted with the B site of the kappa light (L) chain gene enhancer (52). Constitutive nuclear NF- κ B activity appeared to occur only in mature B lymphocytes. In most non-B cells, inactive NF- κ B protein is present sequestered in the cytoplasm with inhibitor proteins termed I κ Bs (3). Activation of the NF- κ B/I κ B complex involves phosphorylation and degradation of I κ B (8,24), allowing for translocation of active NF- κ B complex into the nucleus where it can bind to κ B responsive elements. Activation and nuclear localization can be induced by several agents, including oxidative stress (reviewed in references 2,5,22). NF- κ B has been implicated in transcriptional regulation of a number of cellular genes involved in control of cell proliferation, adhesion, and in immune and inflammatory responses (2,5,22). These include the oncogene *c-myc*, several genes encoding growth factors or interleukins or their receptors, and adhesion molecules such as E-selectin, ICAM-I, and VCAM-I. We demonstrated that the murine *c-myc* oncogene contains two functional κ B sites (16,28). The human *c-myc* gene was found to contain similar κ B elements (25).

The biochemical characterization of classical NF- κ B determined that it is a heterodimer composed of a 50 kDa (p50) and a 65 kDa (p65) subunit. Cloning and sequencing of p50 and p65 led to the discovery that the binding domains of these factors have homology with an approximate 300 amino acid domain of the v-Rel oncoprotein and was thus termed the Rel

homology domain (RHD), hence this family is termed the Rel or NF- κ B/Rel family (21,29,45). In addition to c-Rel, other members of the Rel family have been discovered, including p52 (also called I κ B) (7,42), RelB (46), and the product of the *Drosophila dorsal* gene (53). Rel-related factors bind as hetero- or homodimers that have different activities depending on subunit composition (55). For example, the p65 subunit is able to potentially transactivate promoters driven by κ B elements (31,48). The c-Rel protein, which appears to function in an element specific fashion, transactivates more moderately (31, 35,36,54). RelB is also a potent transactivator but only functions as a heterodimer (15,46). The overall effect within a cell is determined by the balance of dimers expressed, and is specific to the gene of interest. Our preliminary results indicate malignant breast cell lines and primary tissue constitutive express active Rel factors, including c-Rel and p65. Given the potential important role NF- κ B/Rel may play in the control of cell proliferation further study of these factors in breast cancer was proposed in this application.

BODY AND CONCLUSIONS

Specific Aim 1: Quantitate and compare the levels of the individual subunits of NF- κ B/Rel in nuclei of normal and transformed breast cell lines and primary human breast cancer tissue.

Induction of NF- κ B/Rel Activity Parallels Increased Transformation. Stampfer and colleagues have recently established an orderly staging of progression following carcinogen treatment of primary human breast epithelial cells to an immortalized cell (62). The 184 HMEC (human mammary epithelial cell) finite lifespan cells were obtained from reduction mammoplasty tissue. In culture, they senesce around passage 22 (p22). Following *in vitro* exposure of 184 HMEC with benzo(a)pyrene (BP), a clonal isolate 184Aa emerged. This clone displayed slow continuous growth with individual cells displaying loss of proliferative ability. After 20-30 passages, continuous slow growth occurred. Indefinite lifespan 184A1 cells appeared from the 184Aa cells at p9, displaying faster growth, greater refractility, smaller size and growth as single cells *vs* patches. In collaboration with M. Stampfer, we have examined the NF- κ B/Rel levels in nuclear extracts from the finite lifespan 184, early passage conditional immortal 184A1, and fully immortal 184A1 in quiescence (Fig. 1). The resulting EMSA show that the low basal level of binding in the finite lifespan cells increases significantly in the early

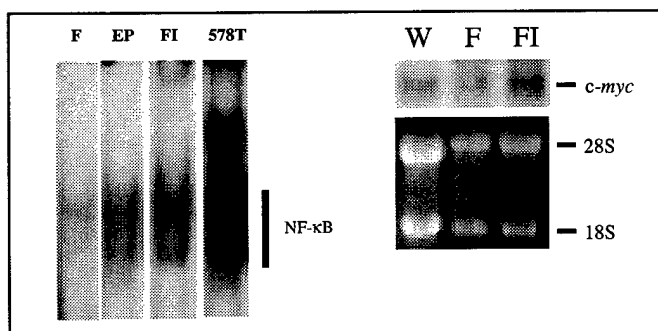


Fig. 1. BP treated HMEC display higher NF- κ B/Rel activity. Left) NF- κ B EMSA from finite lifespan 184 (F), and 184A1 early passage conditional immortal (EP), and fully immortal cells 184A1 (FI), and 578T breast cancer cells. Right) Analysis of c-myc RNA levels in quiescent finite (F) and fully immortal (FI) cells. Ethidium bromide staining indicates equal loading. WEHI 231 (W) RNA added as a control for detection.

passage conditional immortal, and then is increased further in fully immortal cells. The level of binding in the fully immortal cells is lower than that seen in 578T cells (and essentially

equivalent to that seen with nuclear extracts from MCF-10F cells, data not shown). More recently, we have found that *c-myc* mRNA levels in the quiescent finite lifespan and fully immortal cells change commensurately with the increase in NF- κ B, indicating that the factor is functional (Fig. 1). Thus the increase in NF- κ B appears to occur early, by immortalization.

Specific Aim 2: Test the transactivation activity of Rel specific subunits overexpressed in transformed primary tissue in a breast cell line.

We originally proposed to evaluate the effects of transfecting untransformed MCF-10F cells with various subunits of NF- κ B and monitor for functional activity, using markers such as proliferation, or expression of *c-myc*. However, we initially had difficulty in obtaining efficient transfection of this line. Thus, we used the alternative approach of transfecting the 578T cell line. We have found that ectopic expression of c-Rel ablates growth arrest of 578T cells mediated by transforming growth factor β 1 (TGF- β 1). In addition, we obtained chemically transformed MCF-10F cell lines from J. Russo, that display increased transformed phenotype. These have been subjected to analysis for NF- κ B expression, and a direct correlation found. Very recently, we have solved the difficulty of transfecting the MCF-10F cell line by using Fugene. Our studies with the 578T cells and MCF-10F transformed lines are described below.

The Inhibitory Effects of TGF- β 1 on Proliferation of Breast Cancer Cell Lines Are Mediated via Down-Regulation of Aberrant NF- κ B/Rel Expression. Several studies have shown that concentrations of (TGF- β 1) ranging from 0.1-10 ng/ml can inhibit the growth of numerous breast tumor cell lines, including 578T and MCF7. While a recent study has also implicated this factor as an important mediator of tamoxifen-induced apoptosis in MCF7 cells (63), the clinical use of TGF- β 1 has been hindered by the loss of the TGF- β type II receptor, which mediates the effects (64). Data from our lab in B cells and hepatocytes has shown a novel signaling mechanism of TGF- β 1 that involves a decrease in NF- κ B/Rel activity as a result of increased I κ B- α expression (65). We therefore assessed the effects of TGF- β 1 on NF- κ B/Rel expression in the breast tumor cell lines 578T (Fig. 2) and MCF7 (data not shown). TGF- β 1 treatment decreased the level of NF- κ B/Rel binding activity in both cell lines. This decrease could be related to a selective increase in the stability of I κ B- α protein that correlated with decreased phosphorylation. To test the role of the drop in NF- κ B/Rel binding, populations of 578T cell stably transfected with c-Rel were prepared, and individual clones isolated by limiting dilution. The mixed population of transfectants showed modest protection from TGF- β 1-mediated growth inhibition (data not shown) which correlated with maintenance of NF- κ B/Rel binding (Fig. 2). Furthermore, when two of the individual clones were tested, they were found to display very significant resistance to growth arrest mediated by TGF- β 1 treatment as judged by the non-radioactive MTS proliferation assay (upper curves, Fig. 3A) and by incorporation of 3 H-thymidine (Fig. 3B), which correlated with maintenance of c-Rel expression (data not shown). Thus, TGF- β 1 treatment of breast tumor cell lines results in a decrease in NF- κ B/Rel activity and a decrease in cell growth; expression of c-Rel can ablate this growth inhibition. These cell lines will be used to evaluate the effects of NF- κ B on *c-myc* expression and on tumor formation.

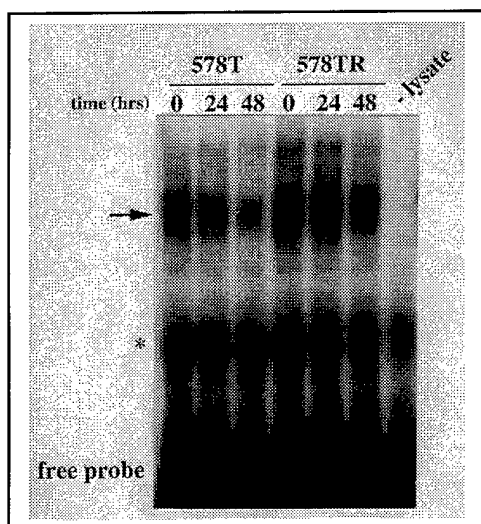


Fig. 2. The effects of TGF- β 1 treatment on nuclear NF- κ B/Rel binding activity. c-Rel transfected 578TR (578TR) and parental 578T (578T) cells were plated at 40% confluence and treated with 2 ng/ml TGF- β 1 for 24 and 48 hrs. Nuclear extracts were isolated and subjected to EMSA using the URE NF- κ B oligonucleotide as probe. Asterisk (*) indicates a nonspecific band that did not change with TGF- β 1 treatment.

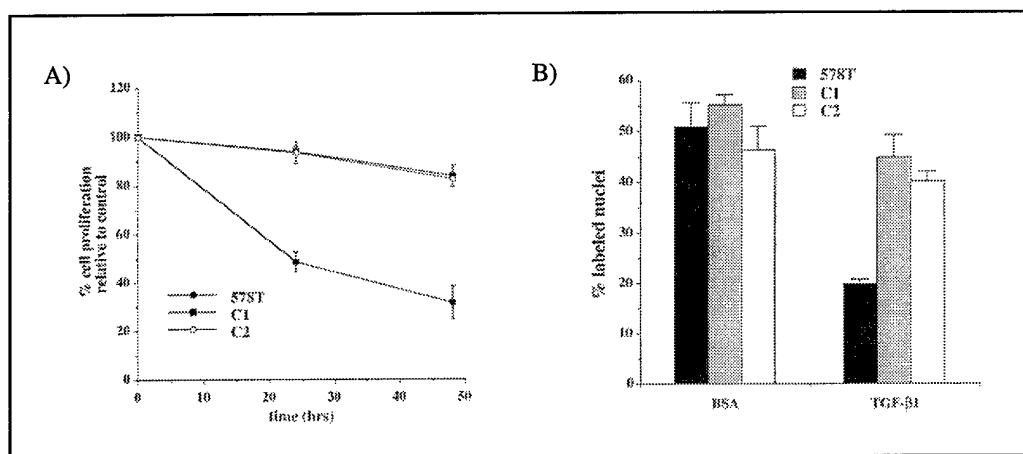


Fig. 3. Ectopic c-Rel expression ablates the effects of TGF- β 1 on growth of 578T clonal isolates.

A) MTS Proliferation assay. Parental 578T and clonal 578TR-C1 and 578TR-C2 cells were plated at 20% confluence and treated with 5 ng/ml TGF- β 1 or BSA as control for 24 and 48 hrs. The effects of TGF- β 1 on growth were measured by MTS assay. Cell numbers are for TGF- β 1-treated cells are given as percent values relative to BSA-treated control cells. B) DNA synthesis. Parental 578T and clonal 578TR-C1 and 578TR-C2 cells were treated in duplicate with 5 ng/ml TGF- β 1 for 48 hrs or BSA as control. Cells were then incubated in media containing 2 μ Ci of [3 H]-thymidine per ml for 6 hours, fixed and exposed for autoradiography. Percent labeled nuclei was determined by visual counting. Mean and standard deviation were determined in two different experiments. Black bars, parental 578T cells; grey bars, clone 1; white bars, clone 2.

Increased Expression of NF- κ B Correlates with Carcinogen-Induced Increase in Transformed Phenotype of MCF-10F Cells D3-1 and BP-1 cell lines were derived from untransformed MCF-10F cells by 24 hour treatment of either DMBA and BaP, respectively (66).

Both cell lines exhibit the malignant characteristics of increased anchorage independent growth, increased chemotaxis and chemoinvasiveness. D3-1 cells exhibit these capabilities to a lesser extent than BP-1 cells (66). Nuclear extracts from D3-1 and BP-1 cells displayed significantly increased NF- κ B binding activity compared to the parental MCF-10F cells (inset Fig. 4). Two bands were seen with the extracts from the D3-1 and BP-1, which co-migrated with bands seen with nuclear extracts from the MCF-10F cells. (The upper band with MCF-10F extract was better seen on a longer exposure.) To verify functional activity, transient transfection analysis was performed with the wt E8 and dm E8 NF- κ B-TK-CAT constructs and SV40- β gal to normalize for transfection efficiency (Fig. 4). The data are presented as the activity of wild type construct relative to that of the dm E8, to obviate differences in transfection efficiencies amongst the lines. The parental MCF-10F cells showed a minimal induction of wt E8 activity over the dm E8 ranging from 1.7-fold \pm 0.6. The D3-1 and BP-1 cells showed an induction of NF- κ B activity of 4.1-fold \pm 1.4 and 11.6-fold \pm 0.2, respectively. Thus, the relative levels of binding and activity correlate directly. Supershift analysis, indicated the upper and lower complexes are composed of classical NF- κ B (p50/p65) and p50 homodimers, respectively (data not shown). Thus, the chemically transformed D3-1 and BP-1 cell lines display increased constitutive functional levels of classical NF- κ B than seen in the parental MCF-10F cells.

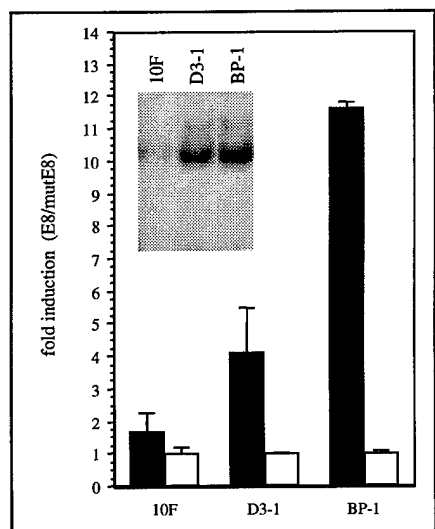


Fig. 4. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF- κ B than the parental MCF-10F cells. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively with 2 μ g wt E8 or dm E8 reporter construct. D3-1 cells were transfected, in duplicate, using 20 μ g of the same vectors by the calcium phosphate method. After 24 hours (for lipofectamine) or 72 hours (for calcium phosphate), extracts were prepared, normalized for protein, and assayed for CAT activity. The values for wt E8 CAT activity are represented as fold induction over dm E8 CAT activity which was set at 1.0 for each cell line.

(Inset) Equal amounts (5 μ g) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabelled URE NF- κ B oligonucleotide as probe.

Specific Aim 3: Monitor the effects of antioxidants known to inhibit NF- κ B/Rel expression on cellular proliferation of transformed mammary cell lines.

We have begun to monitor the effects of treatment with pentoxifylline (PTX) on growth of MCF-7 and 578T cells. In our initial analysis we have found that doses of 1 to 2 mM have significant inhibitory effects on proliferation of these breast cancer cell lines (Figs. 5 and 6).

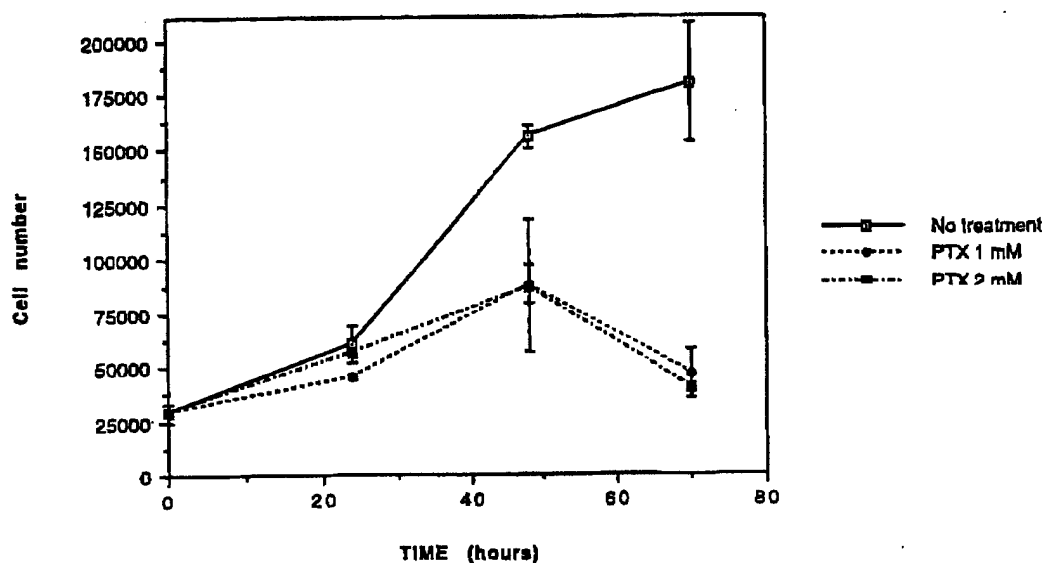


Fig. 5. Effects of PTX on growth of MCF-7 cells.

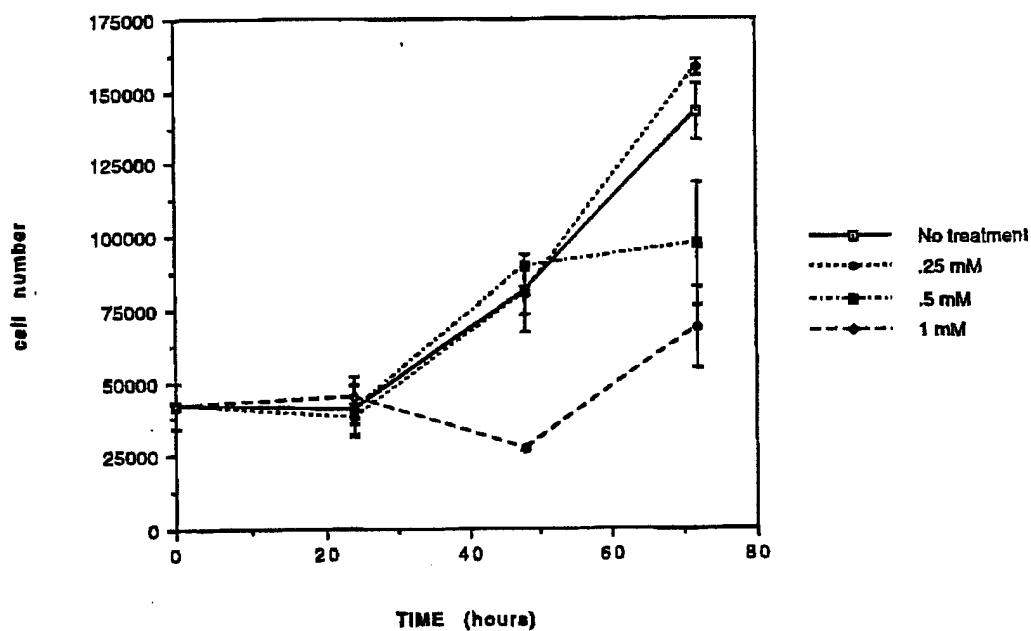


Fig. 6. Effects of PTX on growth of 578T cells.

Specific Aim 4: Use transgenic mice to test the contribution of constitutive NF- κ B/Rel subunit expression in the development of breast neoplasias.

Preparation of MMTV-c-Rel Transgenic Mice. Mouse models have been extremely useful to demonstrate the contribution to breast cancer of specific oncogenes, and anti-oncogenes (91,116,120,129). The mouse mammary tumor virus long terminal repeat (MMTV-LTR) is a hormonally responsive regulatory element that can direct expression of genes to the mammary epithelium as well as to other epithelial tissues. It is activated by corticosteroids and progestins; thus exogenous genes expressed in transgenic mice using the MMTV-LTR as a promoter are upregulated in females with each cycle of pregnancy. In our initial analysis, we have chosen to express the c-Rel subunit under the control of the MMTV promoter. We have subcloned the c-Rel subunit of NF- κ B into the multiple cloning site of the MMTV expression vector to allow us to analyze the positive effects of constitutive expression of NF- κ B/Rel family in mice. The 2.5 kB Eco RI/Hind III fragment from the mouse c-Rel vector pSVsport (kindly provided by T. Gilmore, Boston University) was blunt-end ligated into the MMTV vector. The direction of the insert was confirmed with restriction mapping, and DNA sequencing (by the Molecular Biology Core at BU Medical School). DNA was purified over two CsCl gradients, and the plasmid backbone digested away with SalI and SpeI and the MMTV-LTR, *ras* 5' untranslated sequences, c-Rel cDNA, and the SV40 polyA signal sequence gel purified, electroeluted, and resuspended in 1mM Tris, 0.1mM EDTA for injection. Fertilized oocytes of FVB/N mice were microinjected by Daniel Ladd in the Boston University Transgenic Laboratory (Dr. Katya Ravid, Scientific Director). Six founders have been generated, as judged by Southern blotting of tail DNA (Fig. 7 and data not shown). These animals have been bred and c-Rel transgene-positive homozygous females obtained; these will be used in tumorigenicity studies, as described below.

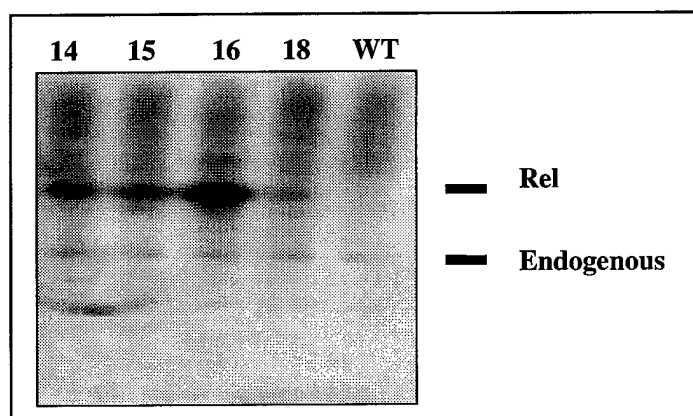


Fig. 7. Southern blot analysis confirming identification of MMTV-c-Rel founder mice.

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